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Similarity of chromosome structure among *Populus tremula* var. *davidiana*, *Populus alba* and their hybrids revealed by FISH karyotype analysis

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Abstract: The genus *Populus* is one of the important tree species in Korean peninsula and many other countries in the world. It represents the model species of forest genomics because it grows fast and reproduces rapidly. In this reason, their genetic characteristics have been well studied and the whole genome has been sequenced completely in some species. However, cytogenetic study of the genus *Populus* has been limited. In the present study, karyotypes of Korean aspen (*P. tremula* var. *davidiana*), Silver poplar (*P. alba*) and their two hybrids, Suwon aspen (*P. tremula* var. *glandulosa*) and Hyun aspen (*P. alba* × *P. tremula* var. *glandulsa*) were analyzed by means of the fluorescence *in situ* hybridization (FISH). Root samples were collected from mature trees in the demonstration forest, located at Suwon, Kyonggi province in South Korea. The fresh root cells were examined by DAPI (4',6-diamidino-2-phenylindole) staining and FISH using 45S rDNA and 5S rDNA probes. As the results, the chromosome compositions of all species were the same as 2n = 38. The karyotype formulas of Korean aspen, Silver poplar, Suwon aspen and Hyun aspen were 28m + 6sm + 4st (2sat), 26m + 10sm (2sat) + 2st, 26m + 12sm (2sat) and 28m + 10sm (2sat), respectively. The four species had one pair of 45S rDNA site and one pair of 5S rDNA site in common with FISH karyotypes. The similarity of FISH karyotypes among four species indicated close genetic relationship and coexistence of their interspecific hybrids. This research will provide genetic information on cytogenetic research of *Populus* and genetic mapping that can be applied to the breeding program of *Populus* in the near future.

Keywords: Karyotype analysis, Fluorescence *in situ* hybridization, Ribosomal DNA site, Poplar, Cytogenetics

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Introduction

Populus is a deciduous tree genus that grows mainly in the temperate zone of the northern hemisphere and planted mainly for biomass production in forestry because of its rapid growth and economic value (Noh et al., 2008). *Populus* is one of the important model tree genus for genetics and whole genome sequencing of *Populus trichocarpa* had been completed (Bradshaw, 2000; Taylor 2002; Tuskan, 2006). In South Korea, Korean aspen (*P. tremula* var. *davidiana* (Dode) C.K. Schneid, synonym *P. davidiana* Dode) is the native species of *Populus*. It grows up to 20 m in height and 1 m in diameter and distributes in Far East Russia, China, Korea and Japan (Chang et al., 2011). Silver poplar (*Populus alba*), only female, is an introduced species from Europe to South Korea in early nineteenth century. Both species have been widely planted as fuel woods and street trees for national forest rehabilitation program (Woo et al., 2005).

Natural hybridization occurs between *P. alba* and *P. tremula* var. *davidiana*, both belonging to the same section of *Leuce* in the genus *Populus*. In 1925, the Japanese botanist Prof. Uyeki found the natural hybrid in Suwon city and gave it a scientific name as *P. tremula* var. *glandulosa*, which is known as Suwon aspen. In addition, a natural hybrid of Silver poplar and Suwon aspen was found and named as *P.* × *tomentiglandulsa* by the well-known botanist, Prof. Tchang-Bok Lee in 1955. Contemporary, an artificial crossing was intensively made between Silver poplar (*P. alba*) and Suwon aspen (*P. tremula* var. *glandulosa*) in the hybrid breeding program led by the famous forest tree breeding scientist, Prof. Shin-Kyu Hyun in South Korea. The artificial hybrid *P. alba* × *P. tremula* var. *glandulosa* F1, known as Hyun-aspen, was released and widely planted especially at hilly, mountainous areas in South Korea (Sun, 2009; Chang et al., 2014). The Korean aspen grows well in the barren mountains, while the Silver poplar shows good growth on the water-rich flats, the Hyun-aspen (artificial hybrid) shows strong hybrid vigor (heterosis) for growth and straightness at the plantations. Recently, the artificial hybrid is used for riparian buffer and biomass production, which is one of the major targets in the breeding program of poplars in South Korea.

Poplar genome sequence project has been promoted as the multi-governmental genome project (2014~2021) for fostering the research and industry on post-genome era in South Korea. In the field of forests, association mapping based on genomic breeding populations is underway for poplars (Wei et al., 2014). Especially, karyotype analysis and chromosome study are the base of genome study. However, cytogenetical research has been limited on the poplars and karyotype analysis using fluorescence *in situ* hybridization (FISH) has not been conducted in Korea aspen, Silver poplar and their hybrids.

The present study aimed to 1) identify the cytogenetical differences among four *Populus* species (*P. tremula* var. *davidiana*, *P. alba*, their natural hybrid variety *P. tremula* var. *glandulosa* and the artificial hybrid *P. alba* × *P. tremula* var. *glandulsa*) by the FISH analysis, 2) compare the cytogenetical karyotypes among Korean aspen, Siler poplar, Suwon aspen and Hyun aspen, and 3) provide cytogenetic information on gene mapping and tree breeding of *Populus* species.

Materials and methods

Plant materials

Root cutting and mini-cutting methods were referenced from Koo et al. (2010). Root cuttings were collected into 5~10 cm in diameter and 20~30 cm long from mature trees of each poplar species (*P. tremular* var. *davidiana*, *P. alba*, *P. tremula* var. *galndulosa* and *P. alba* × *P. tremula var. glandulosa*), and planted in pots with an upper part exposed and covered with vinyl to keep moisture. The pots were placed on culture shelves (fluorescent light in 08:00-24:00) and soaked entirely once every 2 or 3 days.

After 3 to 4 weeks, new shoots began to grow from the root cuttings. When the shoots grew to 15~20 cm, they were cut with pruning scissors leaving about 2~3 cm from the base. The shoots were divided into 3~5 cm mini cuts, and rooting agents were applied. The mini-cuttings were planted in small pots filled with soils. The top of the cuttings was applied by latex emulsion. The mini-cuttings were covered with wire-shaped vinyl to maintain moisture. The mini-cuttings were placed on the growth chamber under fluorescent lights between 8 and 24 hours, and water was regularly applied.

Chromosome preparation

Sampling methods were referenced from Waminal and Kim (2012). The soil and minor debris were removed from the tip of roots collected by brushing and washing. The fresh roots were cut at 9~10 am at the most active period of cell division. At this time, the tip of the root was picked up as thick as possible in green or white color. The remaining roots after the pre-cut were kept in chilled ice-water.

Fixation methods are described in Kato et al. (2004) and Wanimal et al. (2012). The root samples were placed in a 10 mL vial containing 8-hydroxyquinoline solution (0.002M), and stored in a water bath at 12 °C for 5 hours. After that, 8-hydroxyquinoline was discarded and washed twice with Carnoy's solution (ethanol: glacial acetic acid = 3: 1, v / v).

The sample was then stored at room temperature for 2 hours in Carnoy's solution. Finally, the Carnoy's solution was discarded, washed twice with 70% ethyl alcohol solution, and stored in a refrigerator at 4 °C. The fixed roots were used within a month.

The 40 μL of CP (cellulase and pectolyase; 1% pectolyase and 2% cellulase) solution was added to the e-tube. The root samples were stored at 37 °C for 2 hours in an incubator to activate the enzymes, thereby allowing the cell walls to be degraded. After 2 hours, the CP solution was removed by pipetting, 40 μL of distilled water was added, and the solution was kept on ice for 5 minutes. After that, water was removed from the e-tube and 50 μL of Carnoy's solution was added. Root samples in the e-tube were squeezed with a needle. The e-tube was vortexed for 20 seconds and kept on ice for 5 minutes, and centrifuged at 5,000 rpm for 5 minutes to submerge the cells. Then, the e-tube was inverted and discarded the Carnoy's solution, and 20 μL of acetic acid-ethanol (9:1) solution was added. Finally, centrifugation was performed briefly to submerge the solution.

Two slide glasses (76 mm × 26 mm × 1 mm) were prepared for each e-tube, placed in a water bath, and waited until water droplets were formed. The 10 μL of the solution containing the cells was placed on a slide glass with a zigzag manner by a pipette, and then dried in a 40 °C oven. In a dark room, 40 μL of DAPI stock solution (Roche, Germany) in 2 X SSC solution (1:99, v/v) was placed on the cover glass (24 mm × 50 mm). The slide glass was turned over and attached to the cover glass until all of the DAPI solution had spread. A fluorescence microscope (Olympus BX51, 100×, 400×, 1000×) was used to observe the cell division carefully without any missing portions. If there was a good image, mapping was performed to show the same position on the extra slide glass. After that, a fluorescence *in situ* hybridization (FISH) was performed.

FISH karyotype analysis

The sequence of 45S rDNA probe and 5S rDNA probe used for FISH is described by Waminal et al. (2018). The 45S rDNA probe was labeled with AlexaFluor™ 5-dUTP (Invitrogen, USA) and the 5S rDNA probe was labeled with Diethylamino Coumarim-5-dUTP (Invitrogen, USA). The FISH was performed by selecting a sample in which the chromosome was well observed. In a dark room, 40 μL of total staining solution was added to the e-tube (adding 32 μL of FM, 2 μL of 5S rDNA probe, 2 μL of 45S rDNA probe, 2 μL of telomere and distilled water). The e-tube was submerged in a separator and kept in ice.

The 40 μL staining solution was dropped on the cover glass and pressed to spread the solution. The cover glass was heated at 80 °C for 5 minutes with ThermoBrite, and hybridized for 30 minutes at room temperature in a humidified space. The cover glass was removed and washed. The cells were shaken in 2 × SSC solution for 10 minutes at room temperature, for 25 minutes at 42 °C in water bath and for 5 minutes at room temperature by Laboshaker D600 (Labogene, Korea), and then treated with 70%, 90%, and 100% ethyl-alcohol solutions for 3 minutes each. Finally, 40 μL of DAPI-vector shield solution (Vector Lab., Inc., USA) was dropped onto cover glass (24 × 50 mm). The slide glass was turned upside down and attached to a cover glass, waited until the DAPI solution spread throughout, and observed using a fluorescence microscope.

Good spreading image was taken after 1,000 times try using a CCD camera (Leica DFC 365 FX, Germany) attached to a fluorescence microscope. Photographs were analyzed using Cytovision©/GenusTM 7.2 (Applied Imaging, USA) program, and edited using Adobe Photoshop CS6 (Adobe, USA). The best phase was selected to analyze the chromosome length, 45S rDNA sites and 5S rDNA sites, and reliability was improved by repeated images. The morphology of chromosomes is described by Levan et al. (1964). The chromosomes were numbered according to their shape and arranged in order of length.

Results

Karyotype analysis of the four species of *Populus* revealed that all species contained a total of 38 diploid chromosomes (2n = 38). The karyotype formulas of Korean aspen (*P. tremula* var. *davidiana*), Silver poplar (*P. alba*), Suwon aspen (*P. tremula* var. *glandulosa*) and Hyun aspen (*P. alba* × *P. tremula* var. *glandulsa*) were 28m + 6sm + 4st (2sat), 26m + 10sm (2sat) + 2st, 26m + 12sm (2sat) and 28m + 10sm (2sat), respectively (Table 1).

The chromosome length ranged from 1.45 to 4.69 μm in paternal Korean aspen and varied from 1.37 to 5.03 μm in maternal Silver poplar. The chromosome length of natural hybrid, Suwon aspen ranged from 1.26 to 4.53 μm, and that of artificial hybrid, Hyun aspen varied from 1.56 to 5.36 μm. The total chromosome length of Korean aspen, Silver poplar, Suwon aspen and Hyun aspen were 42.91, 47.92, 39.59 and 50.53 μm (Table 1).

Chromosome No. 2 showed a large difference of length among species, ranging from 4.52 μm (Silver poplar) to 2.72 μm (Korean aspen). The difference of chromosome length seemed to signal the hybridization of Silver poplar, Korean aspen and Suwon aspen. The length of the chromosome 2 of Suwon aspen was located between Hyun aspen and Korean aspen, but more closely to Korean aspen.

Table 1. Karyotype analysis of mitotic metaphase chromosomes of Korean aspen (*Populus tremula* var. *davidiana*), Silver poplar (*P. alba*), Suwon aspen (*P. tremula var. glandulosa*) and Hyun aspen (*P. alba* × *P. tremula* var. *glandulosa*)

	Chr No.	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19
Korean aspen	Total arm length	4.69	2.72	2.69	2.67	2.61	2.55	2.48	2.44	2.40	2.35	2.05	1.96	1.83	1.71	1.64	1.60	1.56	1.51	1.45
	Arm type	m	st	sm	sm	m	st (sat)	sm	m	m	m	m	m	m	m	m	m	m	m	m
Silver poplar	Total arm length	5.03	4.52	3.05	2.95	2.89	2.83	2.66	2.63	2.36	2.30	2.18	2.12	2.02	1.96	1.87	1.87	1.76	1.55	1.37
	Arm type	m	sm	sm	m	sm (sat)	m	m	st	m	m	m	sm	m	m	m	sm	m	m	m
Suwon aspen	Total arm length	4.53	2.81	2.52	2.47	2.41	2.33	2.31	2.24	2.09	2.08	1.79	1.61	1.61	1.59	1.56	1.55	1.44	1.39	1.26
	Arm type	m	sm	sm	sm	sm (sat)	sm	m	m	m	sm	m	m	m	m	m	m	m	m	m
Hyun aspen	Total arm length	5.36	3.52	3.49	3.24	2.94	2.82	2.78	2.73	2.53	2.49	2.40	2.32	2.23	2.22	2.10	2.00	1.88	1.72	1.56
	Arm type	m	m	sm	sm	sm	m	sm (sat)	m	m	m	m	m	sm	m	m	m	m	m	m

Arm type: m – metacentric, sm – submetacentric, st - subtelomeric, sat – satellite chromosome

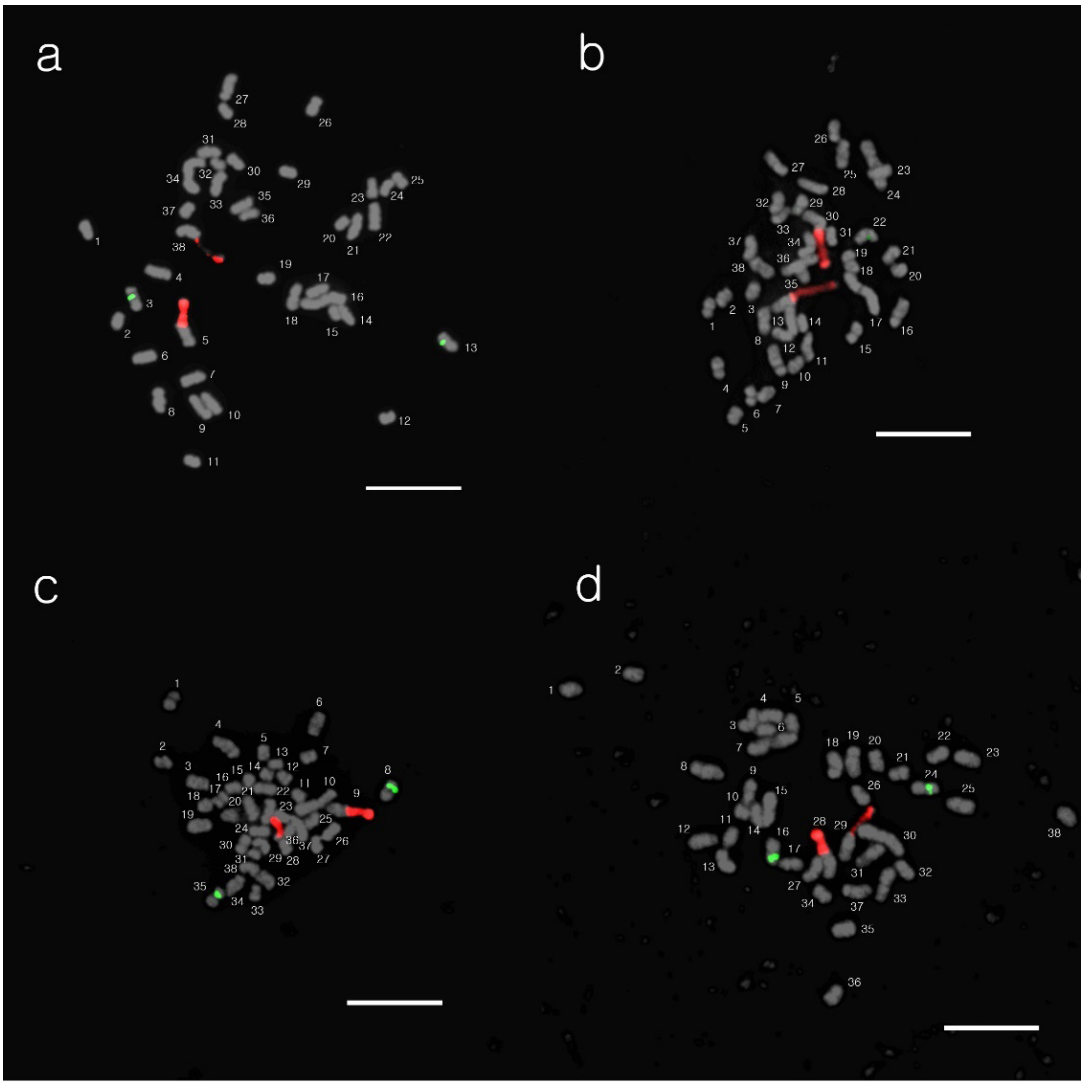

Fig. 1. Fluorescence *in situ* hybridization (FISH) of the 45S and 5S rDNA to chromosomes of four *Populus* species. (a) Korean aspen, *Populus tremula* var. *davidiana*, (b) Silver poplar, *P. alba*, (c) Suwon aspen, *P. tremula* var. *glandulosa* and (d) Hyun aspen, *P. alba* × *P. tremula* var. *glandulosa*. Red signals indicate 45S rDNA sites and green signals indicate 5S rDNA sites. Bar represents 10 μm

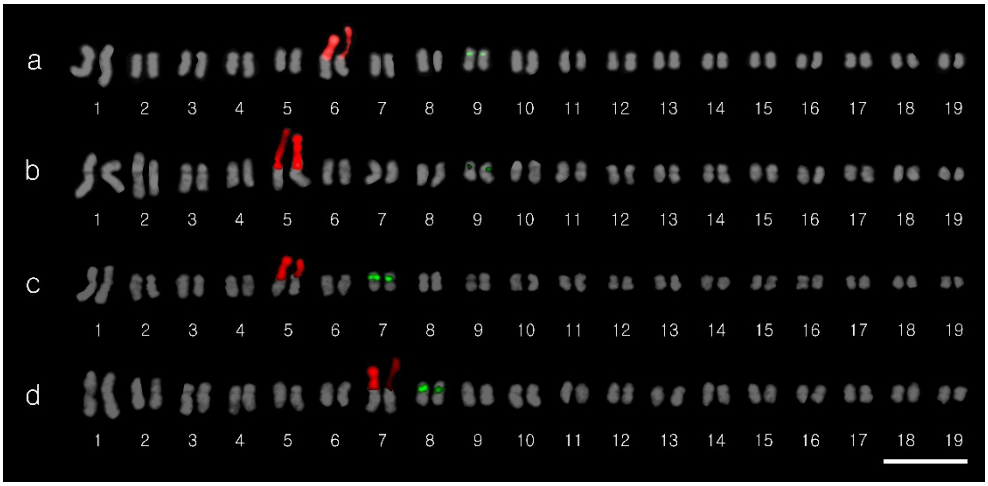

Fig. 2. FISH karyotypic ideograms based on the chromosome morphology. (a) Korean aspen, *Populus tremula* var. *davidiana*, (b) Silver poplar, *P. alba*, (c) Suwon aspen, *P. tremula* var. *glandulosa* and (d) Hyun aspen, *P. alba* × *P. tremula* var. *glandulosa*. Red signals indicate 45S rDNA sites and green signals indicate 5S rDNA sites. Bar represents 10 μm.

The chromosome compositions and arm ratios among four species were similar. They were composed of 26 to 28 metacentric chromosomes, 6 to 12 submetacentric chromosomes and 0 to 4 subtelocentric chromosomes, but acrocentric and telocentric chromosomes were not observed for all species. The range of chromosome arm ratios of Korean aspen, Silver poplar, Suwon aspen and Hyun aspen were 1.03-3.55, 1.08-3.53, 1.06-2.54 and 1.04-2.92 μm (Table 1).

The numbers of 45S rDNA sites and 5S rDNA sites were the same in all species (Figs 1 and 2). The 45S rDNA sites were located at the short arm of chromosome 5 in Silver poplar (*P. alba*) and Suwon aspen (*P. tremula* var. *glandulosa*), chromosome 6 in Korean aspen (*P. tremula* var. *davidiana*), and chromosome 7 in Hyun aspen (*P. alba* × *P. tremula* var. *glandulosa*). The 5S rDNA sites were existed on the short arm of chromosome 9 of Korean aspen and Silver poplar, while those of Suwon aspen and Hyun aspen were located on chromosomes 7 and 8, respectively (Figs 1 and 2).

Discussion

Our karyotype results confirmed that *Populus* species contained a total of 38 diploid chromosomes, and the length of each chromosome was similar among parental species and their hybrids. The most difference of chromosome length was found in chromosome 2. The length of the chromosome 2 of natural hybrid (Suwon aspen) was close to paternal Korean aspen compared to artificial hybrid (Hyun aspen). This result was in well coincidence with Chang et al. (2011), of which morphological analysis suggested that Suwon aspen is closer to Korean aspen than Silver poplar. Also, the similarity of chromosome compositions and arm ratios showed close genetic relationship among Silver poplar, Korean aspen and the hybrids.

Two chromosomes of a homologous pair exchange equal segments with each other during the cell division of meiosis. If the homologous chromosome numbers are correctly assigned, a crossing over or a chromosome rearrangement occur. However, there will be errors in the length of chromosomes because the degree of condensation can vary depending on the progression of cell division (Nkongolo & Mehes-Smith, 2012). In the present study, smaller chromosomes had similar length in the range of 3.49 to 1.26 μm. Liwang et al. (2005) reported the karyotypes of Korean aspen as 2n = 26m (1sat) + 6sm + 6st (1sat), and Silver poplar as 2n = 31m + 4sm (1sat) + 1st (1sat) + 2t. Their results were consistent with our study for the total number of chromosomes and the number of satellite chromosomes, but the chromosome classification was different from ours. In Korean aspen, two more metacentric chromosomes were found in our study instead of two subtelocentric chromosomes. In addition, we found that five less metacentric, two less telocentric, six more submetacentric and one more subtelocentric chromosomes in Silver poplar, compared to Liwang et al. (2005).

Fluorescence *in situ* hybridization (FISH) gives the high accuracy in numbering chromosomes without mismatch in the morphological classification according to the position of centromeres (Peniton et al., 2019) and it could also separate correctly satellite chromosomes to homologous chromosomes. Liwang et al. (2005) did not utilize the FISH method and their accuracy might not be sufficient due to the low resolution when viewing the photographic data. In the present study, the similarity of the occurrence and arm location of 45S and 5S rDNA sites were observed, despite the different chromosome locations

among species. The chromosomes with 45S and 5S rDNAs should be considered as homologous (Peniton et al., 2019). For improving the consistency of chromosomes, more FISH markers should be developed and applied to distinguish each chromosome in *Populus* species that has short chromosome lengths.

Our study is the first attempt to identify a pair of 45S rDNA site of Korean aspen, Suwon aspen, and Hyun aspen. The presence of a pair of 45S rDNA site in the Silver poplar was described by Prado et al. (1996). In the case of 45S rDNA sites, one pair is observed in section *Leuce* like in our study, two pairs in section *Aigeiros* (*P. nigra* and *P. deltoids*) and three pairs in section *Tacamahaca* (*P. balsamifera* and *P.* ×*canadensis*) (Prado et al., 1996; Islam-Faridi et al., 2009). On the other hand, the 5S rDNA sites are one pair in *P. trichocarpa* and *P. nigra* (Ribeiro et al., 2008; Islam-Faridi et al., 2009). This was the same result as our study, revealing that the 5S rDNA sites of all species were found to be one pair.

Karyotype analysis of *Populus* by FISH method is a useful tool for genomics and gene mapping even though it is not easy to obtain good images due to small genome size with many chromosome numbers in poplar species. The number and location of ribosomal DNA loci (45S and 5S rDNA) are one of the major chromosomal characters (Heslop-Harrison & Schwarzacher, 2011). The same number and location of ribosomal DNA loci between Korean aspen and Silver popular shows the similarity of their karyotype. Both are easily crossed with Suwon aspen and Hyun aspen. In contrast, Korean aspen and other *Populus sp.* which have different number and location of ribosomal DNA loci are nearly impossible to be crossed (Woo et al., 2005; Koo et al., 2010). Therefore, the result of this study could be used to predict the compatibility between *Populus spp.* for hybrid breeding.

Furthermore, the FISH karyotyping can anchor the linkage map into physical chromosomes (Kim et al., 2005). Based on this research, follow-up study may construct the cytogenetic map linked to the genetic map of *Populus spp*. In conclusion, this FISH karyotype result can be applied to the hybrid breeding and molecular breeding program of *Populus*.

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